



EXHIBIT "A"

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SMS 201-995: A VERY POTENT AND SELECTIVE OCTAPEPTIDE ANALOGUE
OF SOMATOSTATIN WITH PROLONGED ACTION

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(Received in final form June 30, 1982)

Summary

Stepwise modification of a conformationally stabilised analogue of that fragment of somatostatin which had been thought to be the essential biologically active moiety has enabled us to synthesise the analogue

1 ES → H-(D)Phe-Cys-Phe-(D)Trp-Lys-Thr-Cys-Thr(ol)
code-named SMS 201-995, which in vitro is three times more potent than the native hormone in inhibiting the secretion of growth hormone, which is highly resistant to degradation by pure enzymes and by tissue homogenates, which in vivo in rat and rhesus monkey is (depending on test system) at least 20 times more active than somatostatin, which is much longer acting, and which moreover in both species is much more selective in inhibiting the secretion of growth hormone than that of insulin. The compound is active by several routes of administration including the oral, is well tolerated both in laboratory animals and in man, and is currently undergoing preliminary clinical trial.

It has long been apparent that the most probable conformation of the cyclic part of naturally-occurring somatostatin ((SRIF)) is that of an extended antiparallel β -sheet with the residues Trp₈-Lys₉ at the corners of a β -turn. It was the Salk group who first showed, however, that not all of the native hormone is necessary for expression of the full activity spectrum (1,2) and Veber and co-workers in particular have suggested (3,4) that the essential pharmacophore is most likely the Phe₇-Trp-Lys-Thr₁₀ fragment. Our work, however, shows that the activity of this tetrapeptide fragment can be potentiated to yield highly active analogues with an activity profile of potential clinical interest.

Methods

Peptide synthesis

The analogues were prepared by use of conventional fragment condensation techniques. The final cleavage of the benzyl-type sidechain protecting groups was performed with boron-tris (trifluoroacetate), BtFA, a method which proved superior to other procedures and is also suitable for large scale production. The

peptides were purified over silica gel and duolite, a hydrophobic carrier, and were homogeneous on ILC and HPLC.

Animal Experiments

All experiments in male rats (220 - 280 g) were carried out under Nembutal anaesthesia. The test substances were applied by either the i.m., s.c. or p.o. route or by infusion, as indicated in the Tables and the text. Blood was collected by decapitation but for the infusion experiments it was drawn from the portal vein. Serum or EDTA-plasma (for glucagon determinations) was separated and deep-frozen. Unless otherwise stated, basal hormone secretion was investigated under the influence of the anaesthesia.

For the experiments in primates, chair-adapted male rhesus monkeys were infused through indwelling cannulas with doses of test substance which decreased stepwise every hour. Blood was obtained from the saphenous vein under stress-free conditions. ID50's were derived by log-probit analysis and represent doses necessary to inhibit hormone secretion by 50%.

In vitro experiments

The in vitro secretion of growth hormone followed essentially the method of Vale (5). Dispersed pituitary cells from female IVANOVAS rats were cultivated in monolayers for 4 days. Test substances were incubated, together with IBMX, for three hours and growth hormone in the medium was determined by means of RIA. ID50's from log-probit analysis represent concentrations necessary to inhibit hormone secretion by 50% of the effect of 10^{-7} M SRIF (maximal inhibition). ID50 for SRIF = 3.0 nM.

The in vitro stability of SMS 201-995 was compared with SRIF against degradation by an ultrafiltrate of rat kidney homogenate. Peptides were incubated at 25°C with the ultrafiltrate prepared as described (6) and the quantity of unchanged peptide remaining was determined by means of HPLC from aliquots removed at various time intervals.

Calculations of Conformation

Conformations were calculated with Momany/Scheraga's ECEPP (7) and drawn with Motherwell's PLUTO (8) programs, both heavily modified for interactive use in a DECsystem-20 computer by T.J.P.

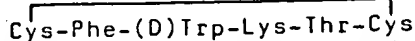
Radioimmunoassays

All hormones were measured by appropriate radioimmunoassays, i.e. rat growth hormone according to (9), rhesus growth hormone with an assay for human growth hormone (10), which shows perfect parallelism of serial dilutions of serum samples with the standard curve, insulin according to R. Haller and P. Marbach (unpublished) and glucagon with the antiserum 30K from the laboratories of Dr. R. Unger and labeled 125 I-glucagon from NEN. SMS 201-995 was measured by an assay which will be described in detail elsewhere. Briefly the antiserum originated from a mouse which had been immunised with the peptide conjugated to hemocyanin. As tracer the [Tyr¹]-analogue of SMS 201-995 was iodinated and purified on HPLC. The assay had a sensitivity limit of 50 pg/ml. Samples could be measured unextracted and the antibody was directed essentially against the sequence 3-6 of the analogue. Calculations were performed according to the program described in (9).

Results and Discussion

Structure Activity Relationship

We chose, as a conformationally adequate surrogate for the full hormone sequence, the cyclic hexapeptide



and proceeded to prepare close analogues with two clear goals in mind: we wanted a compound which would inhibit the secretion of growth hormone (GH) in doses significantly less than those required to inhibit insulin secretion; and we wanted a compound sufficiently stable that it, when properly formulated, could inhibit growth hormone secretion over a therapeutically adequate time span. (SRIF itself has a plasma half-life in man of only about 3 minutes, and can only be given as a continuous infusion).

As can be seen from Table I, our conformationally suitable mimic [1] of the proposed pharmacophore seemed a singularly inadequate starting point for a program of rational drug design - in pituitary cell culture in vitro it had less than 1/1000 the activity of SRIF and was in vivo not much better.

TABLE I

Structures and Biological Activities of some SRIF Analogues
of the Type A-Cys-Phe-D-Trp-Lys-Thr-Cys-B

Compound	Substituents		Relative potencies		
	A	B	GH-inhibition		
			<u>in vivo</u>	¹⁾	<u>in vitro</u>
SRIF	-	-	100	(72) ²⁾	100
1	H-	-NH ₂	1.4	(3)	< 0.1
2	H-	-D-Ser(NH ₂)	3.3	(3)	< 0.1
3	D-Phe-	-NH ₂	165	(2)	4
4	D-Phe-	-D-Ser(NH ₂)	680	(3)	12
5	D-Phe-	-D-Thr(NH ₂)	1160	(2)	47
6	D-Phe-	-Ser(ol)	2800	(3)	19
7	D-Phe-	-Phe(ol)	560	(2)	32
8	D-Phe-	-D-Thr(ol)	1100	(1)	54
SMS 201-995	D-Phe-	-Thr(ol)	7000	(14)	300

1) Pretreatment time 15 min., i.m. application

2) Number of experiments

The addition of a C-terminal residue [2] to the cyclohexapeptide did little to help matters. We therefore investigated ways of incorporating additional structural elements of SRIF into the starting compound, for example an N-terminal amino-acid. One of the amino-acid residues of somatostatin which is thought to be important is phenylalanine 6 (which in the cyclohexapeptide is replaced by Cys). By means of both physical and computer-assisted

model-building (7), we accordingly designed the N-terminal D-phenyl alanyl analogue [3]. The aromatic side chain of this additional amino-acid can occupy at least some of the conformational space available to Phe₆ in SRIF and also protects the disulphide bridge against enzymatic attack. The compound has 4% of the activity of SRIF in vitro but is about equally active in vivo. Addition of the C-terminal amino-acid from [2] to give the octapeptide [4] quadrupled the activity, and a C-terminal D-Thr(NH₂) was still better [5]. The change from the D-amino-acid amide [4] to an amino-alcohol [6] improved the in vivo activity considerably while leaving the in vitro activity largely unchanged. Substitution of Phe(ol) for Ser(ol) in the hope of imitating Phe₁₁ of SRIF improved in vitro activity [7] but gave disappointing results in vivo. The introduction of D-Thr(ol) at the C-terminal [8] resulted in no particular improvement in either test system, but reversion to the natural L-configuration for the C-terminal amino-acid culminated in the synthesis of the title compound, in which the C-terminal Thr(ol) most likely takes the place of the corresponding Thr₁₂ in the native hormone. A possible low-energy conformation of SMS 201-995 is shown in Fig. 1, with the corresponding fragment of SRIF for comparison.

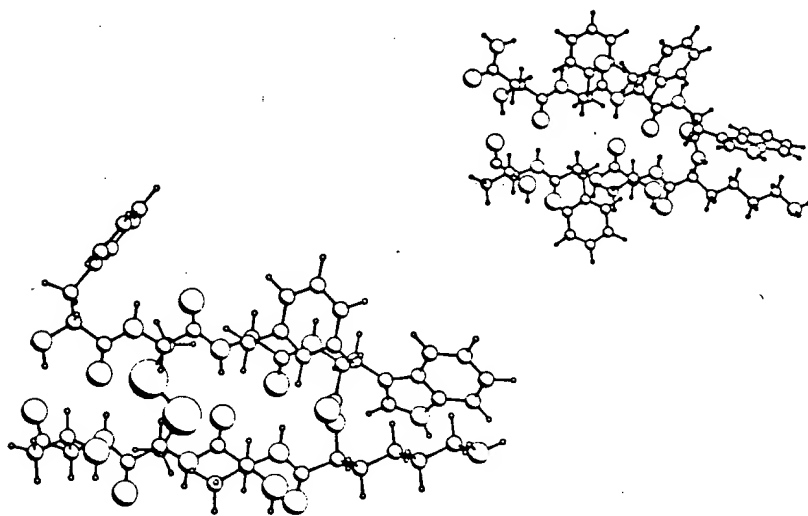


FIG. 1

A possible low-energy conformation of SMS 201-995 with (inset) an identical view of the corresponding fragment of SRIF

Metabolic Stability

Quite early in the synthetic series we decided that we needed a screening test for assessing the stability of the analogues against the actions of degradative enzymes. After a number of experiments with pure enzymes and enzyme mixtures, we settled instead on an

ultrafiltrate of rat kidney homogenate (6) as being capable of degrading most endogenous peptides within minutes. Fig. 2 shows both the remarkable stability of SMS 201-995 compared to SRIF in this test and the variation of the ID₅₀ for GH-suppression in vivo with time for the two compounds. SMS 201-995 has clearly a prolonged duration of action. Measurements of the plasma levels of SMS 201-995 by means of a specific radioimmunoassay after intravenous and subcutaneous administration of a single dose of 10 µg/kg gave plasma half-lives of 10 and 22 min, respectively. SMS 201-995 is also orally active. The ID₅₀ for GH-inhibition in Nembutal anaesthetised rats is 125 µg/kg after one hour.

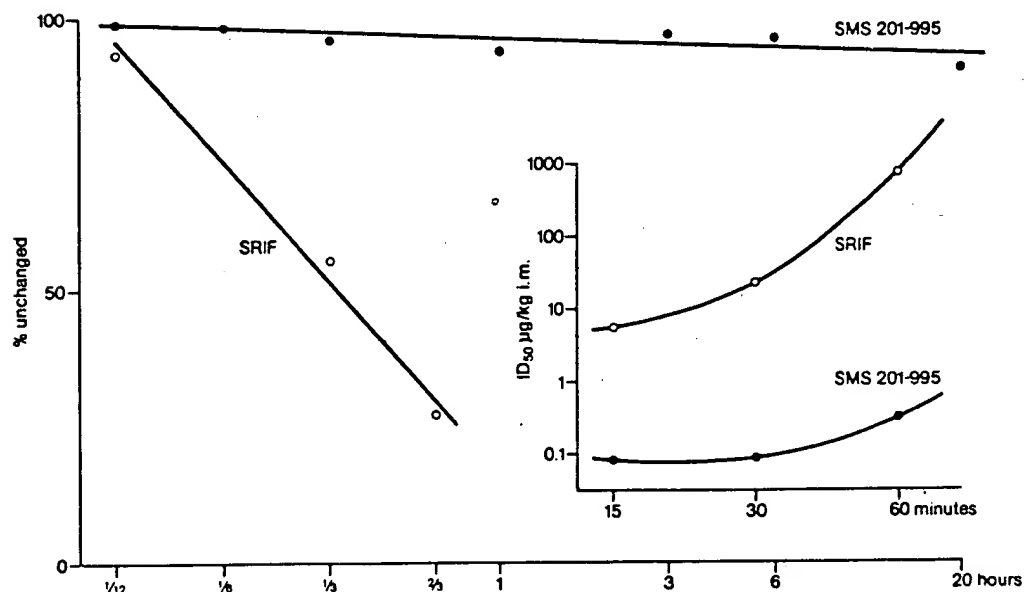


FIG. 2

Stability of SMS 201-995 (●) compared with SRIF (○) against degradation by an ultrafiltrate of rat kidney homogenate (1 out of 3 experiments is shown). Inset the variation of ED₅₀ for inhibition of growth hormone secretion in rats after i.m. injection against pretreatment time (Details see Table II).

Pharmacodynamic effects

The pharmacodynamic effects of SMS 201-995 have been extensively investigated in both rats and rhesus monkeys. In all investigations the compound was compared with SRIF as standard; Table II summarises some of the results obtained in rats. For inhibition of growth hormone secretion, 15, 30, and 60 min. after intramuscular

administration of test substance, the ID₅₀'s are 0.08, 0.08 and 0.34 µg/kg respectively. These doses are rather similar, in contrast to those of SRIF needed under the same conditions (5.6, 26 and 759 µg/kg). The resulting potency ratios of about 70, 300, and 2200 (SRIF = 1) demonstrate not only the high absolute potency of SMS 201-995 but also its much longer duration of action. In the same species, using an identical route of administration and 15 min pretreatment time, SMS 201-995 inhibits glucose-stimulated insulin secretion only 3 times more potently than SRIF and because of its enhanced GH-inhibitory potency is 22 times more GH/insulin specific than SRIF. In contrast, in inhibition of insulin-stimulated glucagon secretion, SMS 201-995 is only 3 times more GH/glucagon selective than SRIF. When SMS 201-995 is given i.v. (15 min before blood-sampling) or s.c. (60 min before blood-sampling) the ID₅₀'s for inhibition of growth hormone secretion are also very low (0.08 µg/kg in both cases), resulting in potency ratios of 800 and 1100 compared to SRIF. The finding that s.c. injection of up to 1 mg/kg SMS 201-995 did not modify insulin secretion indicates that under these experimental conditions this analogue is extremely selective for GH-inhibition in comparison to SRIF.

TABLE II

Comparison of the Pharmacodynamic Effects of SMS 201-995 and SRIF in the Rat

Parameter	Time min.	SMS 201-995 ID50 µg/kg (SEM)	n	SRIF ID50 µg/kg (SEM)	n	Potency Ratio SRIF = 1	Insulin/GH or Glucagon/GH Ratio		
							SMS	SRIF	
<u>GH</u>	i.m.	15	0.08 (0.06-0.11)	14	5.6 (5.2-6.0)	72	70		
		30	0.08 (0.06-0.11)	3	26 (7.9-85)	2	325		
		60	0.34 (0.24-0.49)	3	759 (480-1200)	2	2230		
	infusion	60	0.18/h (0.14-0.23)	4	3.5/h (3.0-4.1)	10	19		
<u>Insulin</u>	i.m.	15 ¹⁾	26 (20-34)	2	77 (66-89)	32	3	309	13.8
	infusion	60	1.4/h (0.92-2.1)	4	6.6/h (5.6-7.8)	8	4.7	7.7	1.9
<u>Glucagon</u>	i.m.	15 ²⁾	0.65 (0.61-0.69)	2	15 (13-17)	14	23	7.7	2.7
	infusion	60	3.5/h (2.1-6.0)	4	17/h (13-22)	7	5	19.4	4.9

¹⁾ 0.5 g/kg glucose i.v., 5 min. before decapitation

²⁾ 1 IU/kg insulin i.v., 15 min. before collection of blood from the portal vein

TABLE III

Comparison of the Pharmacodynamic Effects of SMS 201-995 and SRIF in the Rhesus Monkey

Hormone	SMS 201-995 ID50 $\mu\text{g/kg/h}$ (SEM)	n	SRIF ID50 $\mu\text{g/kg/h}$ (SEM)	n	Potency Ratio SRIF = 1	Insulin/GH or Glucagon/GH Ratio		Selectivity factor SRIF = 1
						SMS	SRIF	
GH	0.04 (0.03-0.05)	3	1.7 (1.2-2.5)	4	45			
Insulin	3.8 (1.2-12)	3	4.9 (3.6-6.7)	4	1.3	100	3	33
Glucagon	0.075 (0.05-0.11)	3	0.8 (0.52-1.3)	4	11	2	0.5	4

When test substances are administered by means of i.v. infusion and hormone measurements are performed after equilibration it may be expected that the ID50's obtained reflect activities which are largely uninfluenced by differing time courses of action. Under such conditions, SMS 201-995 inhibits growth hormone secretion 19 times more potently than SRIF but insulin secretion only 4.7 times more potently. The compound is thus at least 4 times more GH/insulin specific than SRIF.

It may well be that the most relevant data concerning predictability to man are those which result from infusion experiments in primates such as the rhesus monkey (Table III). The ID50 (0.038 $\mu\text{g/kg/h}$) for inhibition of basal GH-secretion is two orders of magnitude less than that required to inhibit basal insulin secretion (3.8 $\mu\text{g/kg/h}$), whereas that for basal glucagon (0.075 $\mu\text{g/kg/h}$) is close to the GH-value. In comparison to SRIF, SMS 201-995 inhibits growth hormone 45 times more potently and is 33 times more GH/insulin specific. To our knowledge, this is the first time that a strict comparison (under identical conditions) of the activities of SRIF and an analogue has been reported for a primate species. We would stress that effects on basal levels of all three hormones were determined in parallel following the infusion of test drug. Such results are likely to represent most closely the situation to be expected in clinical practice.

SMS 201-995 also potentiates the hypoglycaemic effect of insulin, most likely by suppressing glucagon secretion. By simultaneous i.m. injection of varying doses of SMS 201-995 and a fixed i.v. dose of insulin into Nembutal-anaesthetised rats, we have found, for example, that 1 $\mu\text{g/kg}$ of SMS 201-995 in combination with 0.1 IU/kg insulin has the same hypoglycaemic effect as 0.32 IU/kg of insulin alone. This insulin-saving effect could be of value in the therapy of diabetes, quite apart from the advantages one hopes to gain from normalisation of GH levels in such patients.

Like SRIF and other analogues, SMS 201-995 inhibits pentagastrin-induced gastric secretion. In the classical Shay rat the ID50's of SMS 201-995 for inhibition of volume and free gastric acid secretion are 0.7 and 0.4 $\mu\text{g/kg}$ i.m. respectively. These figures compare well with those for SRIF (55 $\mu\text{g/kg}$ and 35 $\mu\text{g/kg}$) when the increase in potency for GH-inhibition is taken into consideration.

Despite being much more potent than SRIF, SMS 201-995 is better tolerated by laboratory animals. In preliminary trials in man, it did not cause any adverse effects up to the highest infused dose

of 50 µg/hour, which is many times higher than the minimal effective dose for inhibition of arginin-stimulated growth hormone release. The results of clinical trials will be published separately.

Conclusion

In conclusion, our stepwise optimisation of a small fragment of endogenous somatostatin has shown not only that it is possible to far surpass the activity of the natural hormone with a synthetic product, but also that both the duration of action and the polyvalent inhibitory activity of somatostatin against various hormones are capable of being manipulated in such a way as better to meet therapeutic goals. We have high hopes that SMS 201-995 will be of value in the treatment of acromegaly, as adjunct therapy particularly in the late-stage vascular complications of diabetes, and in the treatment of various gastrointestinal disorders.

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→ HIGHLY ACTIVE CYCLIC AND BICYCLIC SOMATOSTATIN ANALOGS OF REDUCED RING SIZE